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ENTITLED Localization of the S Layer Gene on the Physical and Genomic Map of Methanococcus voltae

IS APPROVED BY ME AS FULFILLING THIS PART OF THE REQUIREMENTS FOR THE

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Localization of the S Layer Gene on the Physical and Genomic Map of *Methanococcus voltae.*

by

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Thesis

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Introduction

Surface layer (S layer) proteins or glycan subunits, found in an increasing number of bacteria and archaea(10), comprise 5 to 10% of the total cell protein and have potential biotechnological uses as ultrafiltration membranes(6). These self-assembling S layer proteins form the S layer which resides external to the cell wall(11). This protein layer is considered to be the simplest biological membrane(9). There are three proposed functions of an S layer (i) a barrier against external or internal factors, (ii) a framework involved in maintaining the shape of the cells in organisms which possess no rigid cell envelope, and (iii) a promoter for cell adhesion and surface recognition(11).

Surface layers may be the only component of the cell wall, therefore shaping the protoplast(9). This is seen in the Archaea, one of the three domains recently determined by 16S ribosomal RNA(15).

Archaea

The Archaea, Eucarya, and Eubacteria are the three domains as determined by comparitive sequence analysis of 16S ribosomal RNA.(15) The Archaea are a unique biological group containing some features characteristic of the Eucarya and others characteristic of Bacteria. For example, Archaea promoter structure resembles

that of eucarya (16), while the linkage of proteins in the Archaea resemble bacterial operons(2) .

Methanogens

One group of organisms contained in the Archaea, the Methanogens, can utilize simple compounds such as carbon dioxide, acetate, formate, methanol, methylamines, and carbon monoxide as a carbon source(for review see Bhatnagar). Methanogens inhabit a wide variety of environments including the rumen of animals, lakes, ponds, paddy fields, and sewage sludge(5). Methanogens have potential uses in the field of biotechnology including biogas production, water waste treatment, and as a source of thermostable enzymes. To utilize Methanogens for biotechnology, more needs to be known about their biochemistry and genetics. The S layer proteins may be a possible model to study the synthesis and secretion of extra cellular proteins (9), and the S layer gene may be a possible source to study transcription and translation in Methanogens.

M. voltae is a marine methanogen which can utilize CO_2/H_2 or formate as a carbon source. While all other Methanogens are autotrophic, *M. voltae* is not , requiring acetate, leucine, and isoleucine for growth in minimal media(14). *M. voltae* requires sodium, with optimal growth around .5M NaCl. Studies on the protein subunits of the S layer conclude that the molecular weight is 76,000 and that the subunits are of hexagonal symmetry with

center to center spacing of ten nanometers. It is believed that correct assembly and interaction of the S layer proteins in forming the S layer are essential to the structural integrity and viability of the cell, although little is known about the synthesis, secretion of the S layer proteins or the function of the S layer itself. The goal of this project was to map the S layer gene on the physical and genomic map of *M. voltae* , to assist further study of this gene.

Please note that although previously published as the *atp P* gene, encoding for a P type ATPase, it has been recently discovered that the sequence obtained is the S layer gene of *M. voltae* . Therefore, all further references in this thesis to this published sequence will be in reference to the S layer sequence(4).

Materials and Methods

Bacterial strains and growth conditions

M. voltae PS (DSM 1537) was grown in defined media(14), supplemented with 0.1% yeast extract. The cultures were grown in a volume of 500 mL in a one liter Wheaton bottle with a pressurized atmosphere of H_2/CO_2 (80 and 20%, respectively) at 30°C.

Isolation of total DNA in agarose plugs

The following procedures are a modification of Sitzman and Klein(8). 500 mL cultures were grown to early to mid stationary phase, and were precipitated by centrifugation at 8000x g for ten

minutes at 4 °.C The cells were washed in 15 mL of stabilizing buffer (NTEE: 0.4 M NaCl, 0.1 M Tris-HCl, 0.1 M ethylenediaminetetraacetic acid (EDTA), 0.01 M N,N, N',N' tetraacetic acid (EGTA), pH 8.0). Cells were precipitated by centrifugation again and resuspended in 5 mL NTEE. Pronase E was added to low gelling temperature agarose (Seaplaque) in NTEE to a final concentration of 5 mg/ml Pronase E in 1% low gelling temperature agarose. This was then mixed in a 1:1 (vol:vol) ratio with the resuspended DNA. The mixture was pipette into a plug mold. Each plug contains 100 ul of the cell mixture. The mold was placed on ice until the plugs solidified. The plugs were pushed out of the mold into a lysis solution which contained 1.0 % Na Lauroyl Sarcosine in NTEE supplemented with 0.5mg/ml. Pronase E (overnight 37 ° C with two changes of the buffer). The plugs were then washed in 0.05 M EDTA pH 8.0 at 4 ° C for three to four days changing the solution daily. The plugs were stored in fresh solution at 4 °C until preparation for digests. All handling of the plugs was done with alcohol sterilized glass pipettes, glass coverslips, and plastic spatulas. No metal of any kind comes in direct contact with the plug, due to the DNA sensitivity to metals(12).

Digestion of Chromosomal DNA

Plugs were equilibrated in TE overnight, and for one hour in water. Plugs were then preincubated in 400 ul of enzyme reaction buffer for twenty minutes. 200 ul of fresh buffer was added with

80 to 200 units of enzyme (New England Biolabs). Figure 1 contains the enzymes used, their recognition sequence, and units of enzyme added. The reactions were carried out under conditions stated by the manufacturer New England Biolabs with gentle shaking. An additional 20 to 100 units of enzyme was added about 18 hours after the initial addition. After reactions were complete, the plugs were equilibrated in 1 X TAFE electrophoresis buffer (20 X TAFE: .2M Tris, .01M EDTA, .085 M Acetic Acid).

Pulse Field Gel Electrophoresis

Pulse field gel electrophoresis (PFGE) (7) was used to separate the DNA fragments, using the Transverse Alternating Field Electrophoresis (TAFE) apparatus (Beckman). Electrophoretic conditions, modified from Crete (3), are contained in figure 2. All gels were run in 1X TAFE buffer with the temperature held constant at 10 °C with a direct buffer circulation through a cooling waterbath(Beckman).

Southern Transfer

DNA from agarose gels from TAFE-PFGE were stained with Ethidium Bromide and photographed under u.v. light. The gels were treated for 20 minutes with .25 M HCl, denatured 2 X 15 minutes in .4 M NaOH. The DNA was transferred with 0.4 M NaOH onto a Nytran membrane (Schleicher and Schaell) through capillary action. After transfer the membranes were washed in 2 X SSC (20 X

SSC: 3.0 M NaCl, 0.30 M NaCitrate pH 7.0), allowed to air dry, and baked at 80 ° C under vacuum for 2 hours.

Preparation of DNA probes

The probe was prepared by a Pst I digest of the cloned *M. voltae* gene resulting in a 523 bp fragment. The DNA sequence of the S layer gene is contained in figure 3 with the underlined area representing the probe. The fragment was cut from the gel and isolated by the freeze-squeeze method(3). The DNA was then labeled by the Genius nonradioactive DNA labeling and detection kit (Boehringer Manheim Biochemicals).

Hybridization and Immunological Detection

Hybridization and immunological detection was carried out according to the Genius nonradioactive DNA labeling and detection kit.

Results

M. voltae cultures were grown to early-mid stationary phase (1-2 X 10^9 cells per milliliter) (8)and resuspended to 2.5-5 X 10^{10} cells per milliliter in 1% low gelling temperature agarose. All subsequent reactions were completed in agarose to protect the chromosome from sheering. The restriction enzymes do not readily diffuse though the agarose, therefore high concentrations of DNA

and enzymes are necessary to detect the DNA by Ethidium Bromide staining and subsequent Southern hybridization

The enzymes Apa I, BamH I, Bcl I, Bgl II, Eag I, Pvu I, and Sac II, were used by Sitzman and Klein to create the physical and genomic map of *M. voltae*. These enzymes produced 4 to 16 well resolved DNA fragments which allowed them to construct the physical and genomic map shown in figure 4 (8). The enzymes Eag I and Pvu II did not digest well, resulting in partial digests. Therefore these enzymes were not used for this project.

Pulse field gel electrophoresis , specifically transverse alternating field electrophoresis, was used to separate out DNA fragments of 940 kb to 4 kb in length. The principle of pulse field gel electrophoresis is to expose the DNA fragment to two alternating electrical fields. This causes the DNA to reorient, with smaller fragments reorienting faster and therefore migrating further. The time each electrical field is applied to the DNA is referred to as pulse time. As the pulse time increases, the larger DNA molecules are able to reorient and move into the gel. Pulse times were experimentally determined following the procedures described by Crete (3). The 45 second pulses for 8 hours followed by 30 second pulses for 8 hours were found to be most important for maximum separation of the DNA fragments ranging in size from 380 kb to 24kb. In the absense of one of these pulse times, the DNA fragments are condensed and no single bands are obtained in this region.

After obtaining well separated fragments, the DNA was transferred to a Nytran membrane and probed with the Pst I fragment (523 kb) of the S layer gene. Figure 6 highlights the bands obtained by BamH I, Bgl II, and Sac II in a 1% agarose gel. Figure 7 shows the membrane of the gel in figure 6, probed with the Pst I fragment of the S layer gene.

Discussion

The data obtained and summarized in figure 5 indicate the location of the S layer gene to be between positions 1400 and 1500 on the physical and genomic map contained in figure 4. The His A gene is known to reside between map positions 1410 and 1500 (Klein personal communication). There are no other markers mapped within this region that can be used to further determine the position of the S layer gene. Further studies to determine the location of the S layer gene with respect to the His A will involve identifying a large DNA fragment containing both genes, digest this large fragment with various enzymes and probe the DNA with both the His A and the S layer gene. This method should allow an exact determination of the location of the S layer gene with respect to the His A gene on the *M. voltae* physical and genomic map.

Conclusions

The S layer gene was mapped on the physical and genomic map by the use of transverse alternating field electrophoresis. A

full protocol was developed by modification of published methods. DNA isolation was modified to decrease the amount of broken DNA and background. Digestion conditions were altered to increase the band intensity and clarity. Electrophoretic conditions experimentally determined following the procedures described by Crete(3), allowed maximum separation of bands with little background.

Although membranes will be left for the lab for future probing, they will not last more than a few months. The explicit protocols on how to isolate, separate, and probe chromosomal DNA will enable the lab Konisky to readily map any gene that may be discovered in the future.

Appendix

Figure1: Table of restriction enzymes used, amount used, and the recognition sequence. The size range of fragments and the number of bands obtained by the digestion of *M. voltae* chromosomal DNA are summarized.

Figure 1
Table 1: Summary of Restriction Enzymes

Enzyme	Units of Enzyme	Recognition Sequence	Size Range of Fragments	Number of Bands
Apa I	200	GGGCC/C	420-13 kb	16
BamH I	200	G/GATCC	710-24 kb	12
Bcl I	100	T/GATCA	940-90 kb	6
Bgl II	80	A/GATCT	800-5.5 kb	6
Sac II	200	CCGC/GG	310-4 kb	17

Figure 2: Table two contains the optimal electrophoresis conditions for a 1% agarose gel in the Transverse Alternating Field Electrophoresis System. The gels were run for a total of 43 hours with the pulse times and durations as indicated.

Figure 2
Table 2: Electrophoresis Conditions

Pulse times (seconds)	Voltage	Duration (Hours)
60	150	3
45	150	8
30	150	8
15	150	8
10	150	8
5	150	8

Figure 3: Sequence of the S layer gene(4). The underlined area represents the Pst I fragment (5.23 kb) used as the probe in hybridization experiments.

Figure 3:

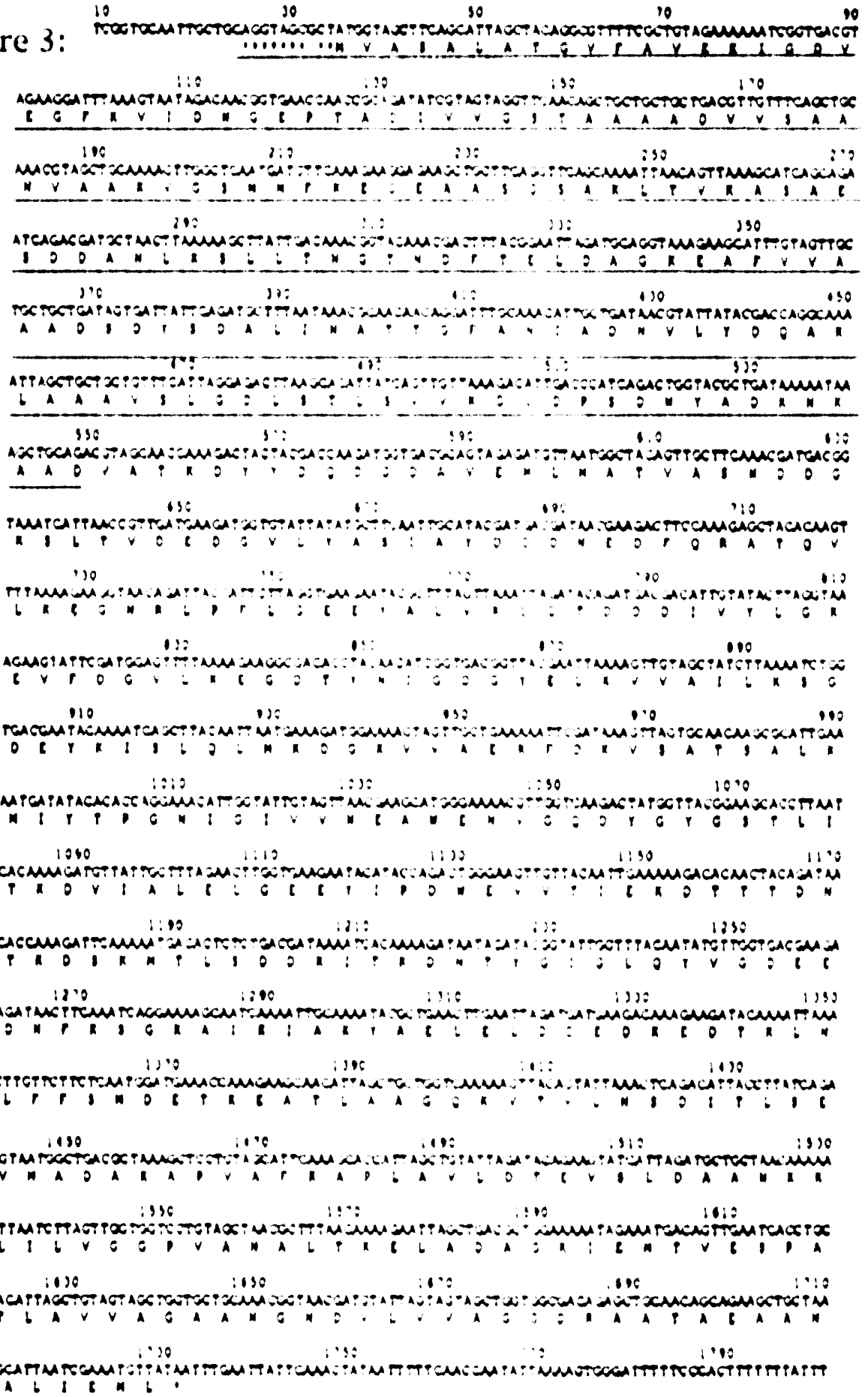


Figure 4: Physical and genomic map of *M. voltae* as determined by Sitzman and Klein(8).

Figure 4:

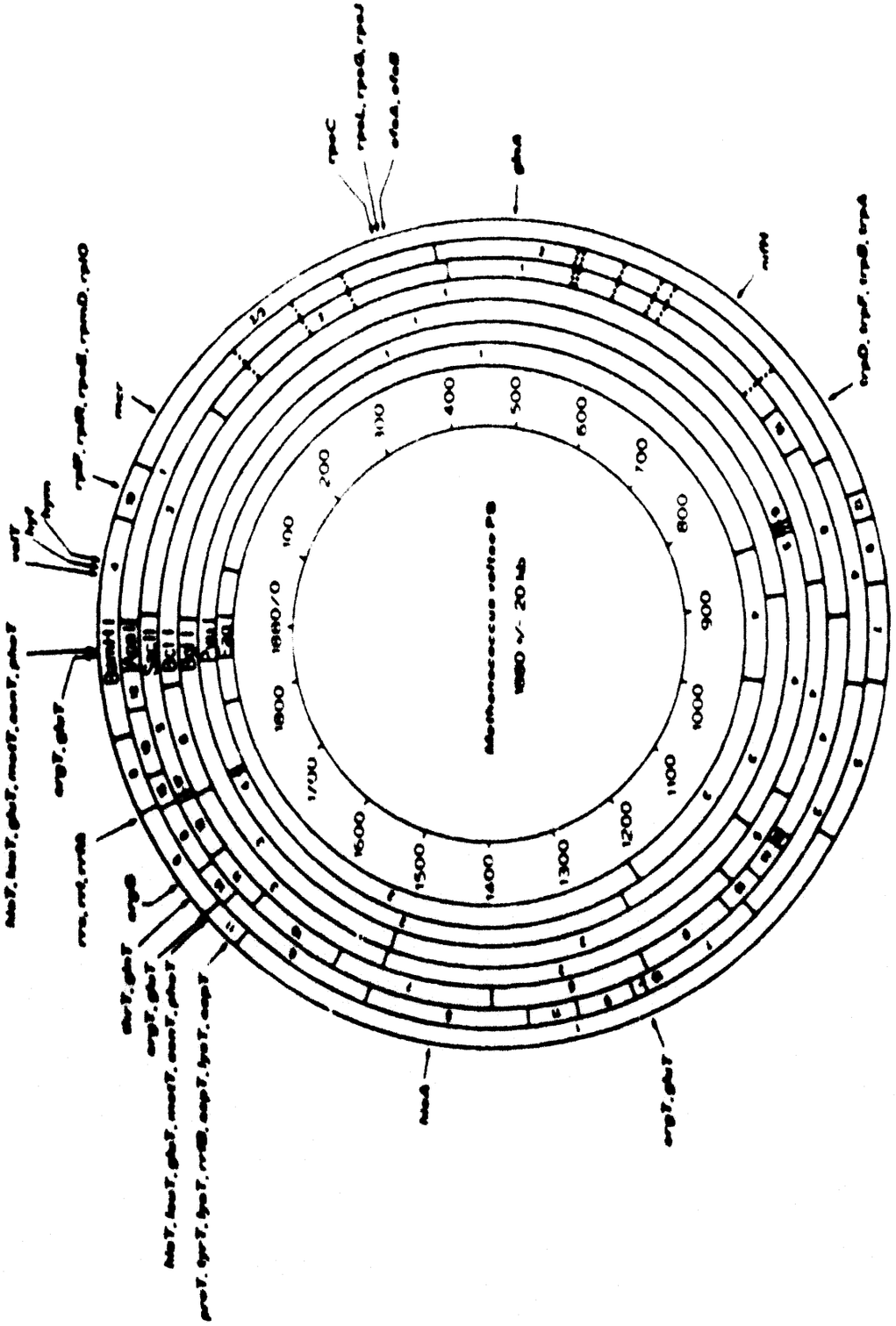


Figure 5: Physical and genomic map from 1300 to 1500 (see figure 4). This figure gives an estimate of relative positions of restriction enzyme fragments that hybridize with the S layer probe and how they related to each other. They restriction fragments that hybridized to the probe are underlined. Ba 1 denotes the largest fragment from the BamH I restriction digest (710 kb). Bc 2 and Bg 2 represent the second largest fragments of Bcl I and Bgl II, respectively. Within this region, Sac II produces two DNA fragments labeled Sa 8 and Sa 7, and Apa I produces three DNA fragments labeled Ap 9, Ap 11, and Ap 5.

Figure 5:

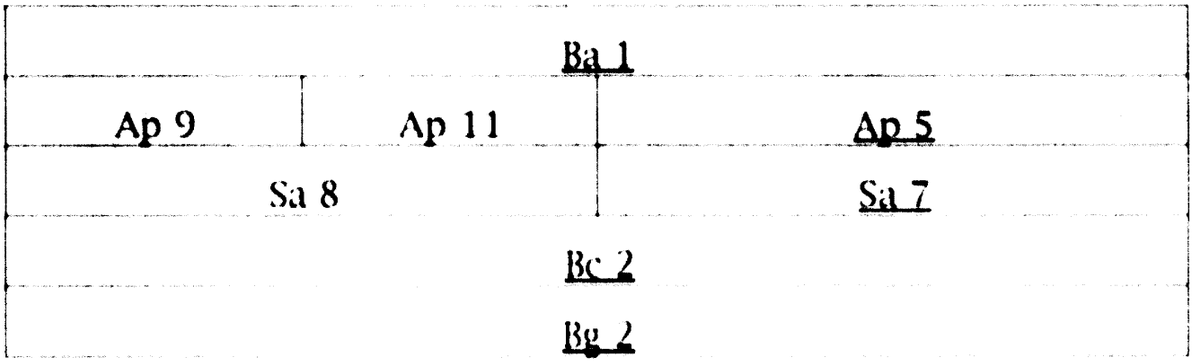
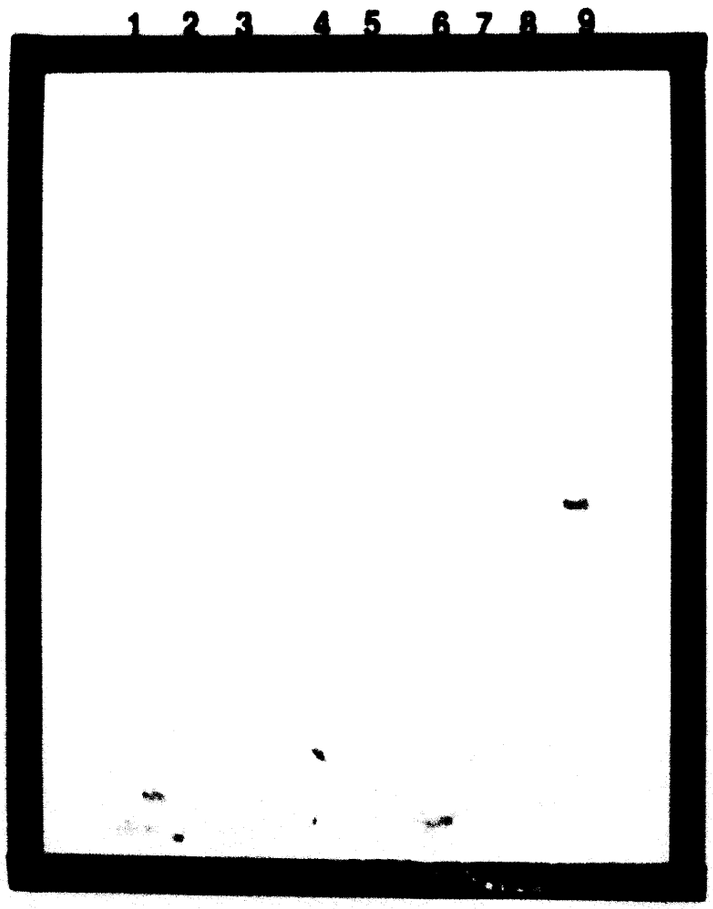


Figure 6: 1% agarose gel of *M. voltae* chromosomal DNA cut with various restriction enzymes. Lane 1: Molecular weight standards(Beckman). Lane 2 and 3 : Apa I digest. Lane 4: BamH I digest. Lane 5 and 6: Bgl II digest. Lane 7, 8, and 9: Sac II digest. The Apa I enzyme is believed to be contaminated due to the unexpected fragments which are different from the results obtained from Sitzman and Klein. (8) The enzyme was reordered and the results were the same.

Figure 6

Figure 7: Southern blot analysis of a 1% agarose gel. Lane 4: hybridization to BamH I 710kb fragment (Ba 1). Lane 5 and 6: Bgl II: Hybridization to Bgl II 385 kb fragment.(Bg 2). Lane 7, 8, 9: Hybridization to Sac II 145 kb fragment (Sa7). These results were obtained in three hybridization experiments for Sac II, and in more than five experiments for BamH I and Bgl II.

Figure 7:



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